Journal of Biomolecular NMR, 2 (1992) 639–646
ESCOM

J-Bio NMR 086

Sequential assignment of the backbone nuclei (^{1}H, ^{15}N and ^{13}C) of c-H-*ras* p21 (1–166).GDP using a novel 4D NMR strategy*

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Received 3 August 1992
Accepted 2 September 1992

Keywords: 4D NMR; c-H-*ras* p21; GDP; GTP; Sequential assignments; Backbone nuclei

SUMMARY

The c-H-*ras* p21 protein is the product of the human *ras* proto-oncogene, a member of a ubiquitous eukaryotic gene family which is highly conserved in evolution. These proteins play an important role in the control of cellular growth. We report here the sequential assignment of the backbone nuclei in a truncated form of the 21-kD gene product, using our recently proposed 4D NMR strategy (Boucher et al., 1992). These assignments are the first step towards a full investigation of the structure, dynamics and interactions of wild-type and oncogenic *ras* p21 using NMR spectroscopy. Some of the data were presented at the 33rd ENC held at Asilomar, California, U.S.A., in April 1992.

Ras genes are amongst the most widespread of all the oncogenes found in human tumours (Barbacid, 1987; Bos, 1989). They encode highly related 21 kD (p21) guanine nucleotide-binding proteins which are located on the inner surface of the plasma membrane. Although their exact function is still unknown, they are believed to play a critical role in the control of cellular growth by switching between the biologically active GTP and inactive GDP forms of the protein. Several high-resolution crystal structures of *ras* proteins complexed to either GDP or stable GTP analogues have recently been reported (De Vos et al., 1988; Pai et al., 1989, 1990; Brunger et al., 1990; Krengel et al., 1990; Milburn et al., 1990; Tong et al., 1991). However, efforts to crystallise com-

* Supplementary material is available from the corresponding authors: One table containing the complete resonance assignment of c-H-*ras* p21 (1–166).GDP.

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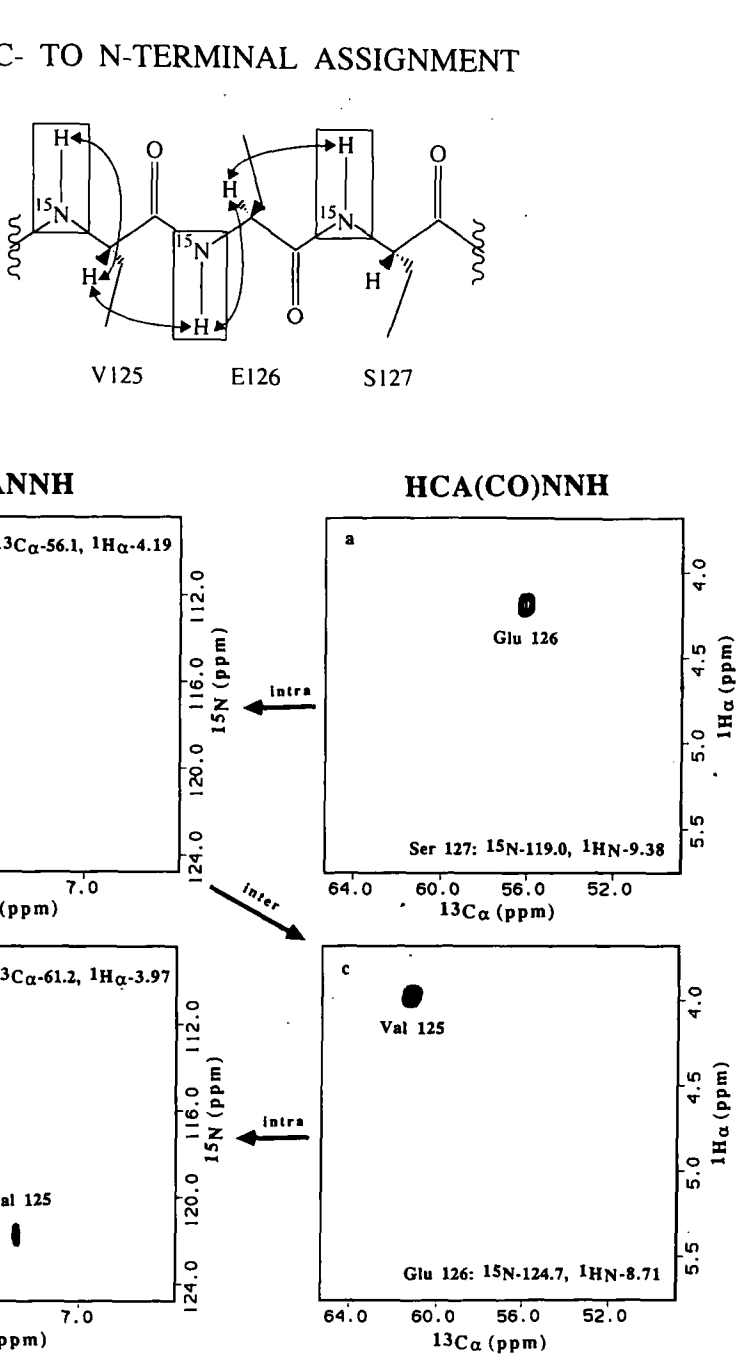
0925-2738/$ 5.00

plexes with proteins which modulate *ras* activity (e.g., GAP or NF1; Trahey and McCormick, 1987; Gibbs et al., 1988; Martin et al., 1990; Ballester et al., 1990; Xu et al., 1990) have been unsuccessful. Detailed studies using NMR may therefore be useful for probing the structure and dynamics of *ras* proteins and their interactions with either GAP, NF1 or other molecules that control *ras* function. Two distinct mechanisms of GTP hydrolysis have recently been proposed (Pai et al., 1989; Prive et al., 1992), but structural information is lacking because the catalytic loop L4 is poorly defined in the structures determined by X-ray diffraction methods. Again, NMR studies of the structure and dynamics of *ras* have the potential to provide insight into the mechanism of GTP hydrolysis and may aid in understanding differences between the wild-type and oncogenic proteins. For any detailed NMR studies of the structure, mechanism or dynamics of the protein, sequence-specific assignments are a prerequisite.

Previous NMR studies of c-H-*ras* p21.GDP, in which specifically labelled amino acids (^{15}N, ^{13}C and ^{2}H) had been incorporated, allowed some assignments to amino acid *type* (Campbell-Burk, 1989; Campbell-Burk et al., 1989; Redfield et al., 1990; Yamasaki et al., 1989, 1992; Schlichting et al., 1990). Although in our initial attempts to assign c-H-*ras* p21.GDP we used the intact protein (189 residues), these attempts were plagued by problems of sample instability, probably resulting from aggregation due to oxidation of cysteine(s) in the carboxy-terminal region of the protein. Light-scattering data showed that, at the concentrations required for NMR studies, the intact protein formed oligomers whereas a truncated form, residues (1–166), appeared to be monomeric. The $^{1}H_N$ and ^{15}N-chemical shifts of glycine residues in the guanine nucleotide-binding domain were found to be very similar in the two forms of the protein, consistent with their similar biochemical properties (John et al., 1989). We have therefore used c-H-*ras* (1–166) for all subsequent NMR studies.

Recently, several 3D heteronuclear triple resonance NMR methods specifically aimed at obtaining sequential resonance assignments of larger proteins (> 150 residues) have been developed (Ikura et al., 1990; Kay et al., 1990). These methods formed the basis for the design of our recently reported 4D NMR experiments, which provide a simpler sequential assignment strategy (Boucher et al., 1992a,b). Intra-residue (and many sequential) assignments are obtained from either a 4D version of a previously published 3D NMR experiment called H(CA)NNH (Montelione and Wagner, 1990; Kay et al., 1991) or a new HNCAHA experiment (Boucher et al., 1992b; Clubb et al., 1992; Wittekind et al., 1992). Sequential assignments are based on a complementary 4D NMR experiment, which we call HCA(CO)NNH, where the magnetisation is specifically transferred via the ^{13}CO group whose chemical shift is not measured. These 4D NMR spectra allow one to make

Fig. 1. An illustration of the carboxy- to amino-terminal assignment of the L8 loop of c-H-*ras* p21 (1–166).GDP. 4D HCANNH and HCA(CO)NNH spectra were recorded under identical conditions on a 1.5 mM sample of c-H-*ras* p21 (1–166) uniformly labelled (>90%) with ^{13}C and ^{15}N, at pH 6.5 and 30 °C in 90%H_2O/10%D_2O, 20 mM Tris-maleate-d_{15}, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.01% sodium azide and 30 μM GDP. A total of 32 (t_1) × 12 (t_2) × 10 (t_3) × 512 (t_4) complex points were accumulated in ~96 h to give acquisition times of 12.8, 4.8, 10.0 and 63.4 ms in t_1, t_2, t_3 and t_4, respectively. The ^{1}H carrier was positioned on the water resonance whose intensity was reduced in the final spectrum by baseline correction in the t_4 time domain (Marion et al., 1989b). The spectrum was first processed using conventional Fourier transforms in both t_4 and t_1. The low-frequency portion of the spectrum was discarded after the first transform. Processing in the t_2 and t_3 dimensions was achieved using a two-dimensional maximum entropy algorithm (Laue et al., 1986) to give a final data matrix of $f_1(64) \times f_2(64) \times f_3(64) \times f_4(512)$ real points; each dataset took ~14 h to



process on 16 transputers in a Meiko Computing Surface (Boucher et al., 1991, 1992a). All spectra were analysed, plotted etc. using the FELIX package from Hare Research. Panels (a) and (c) show specific sequential planes ($f_1 = {}^1H_\alpha$ and $f_2 = {}^{13}C_\alpha$) at particular ($f_3 = {}^{15}N_{+1}$ and $f_4 = {}^1H_{N+1}$) chemical shifts from the HCA(CO)NNH spectrum. Panels (b) and (d) show specific sequential planes ($f_3 = {}^{15}N$ and $f_4 = {}^1H_N$) at particular ($f_1 = {}^1H_\alpha$ and $f_2 = {}^{13}C_\alpha$) chemical shifts from the HCANNH spectrum. The assignment strategy is discussed in the text. Unlabelled cross peaks are due to partial overlap with adjoining planes.

a *simultaneous* connection to both the $^1H_\alpha$ and $^{13}C_\alpha$ nuclei, thus alleviating problems of overlap found in an assignment based on two 3D NMR spectra (e.g., HNCOCA and HNCA, Bax and Ikura, 1991). In this paper we present a virtually complete sequential assignment of the backbone nuclei of c-H-*ras* p21 (1–166).GDP using our 4D NMR approach.

Figures 1 and 2 show 2D planes obtained from the two different 4D NMR spectra. They illustrate the sequential assignment process in a portion of the L8 loop (Tong et al., 1991). In this example, an assignment from the carboxy- to amino-terminal direction proceeds by beginning at the (^1H_N, ^{15}N) coordinates of Ser^{127} while viewing the ($^1H_\alpha$, $^{13}C_\alpha$) 2D plane in the HCA(CO)NNH spectrum. Since this experiment exclusively detected the *inter*-residue cross peaks, we obtained the ($^1H_\alpha$, $^{13}C_\alpha$) shifts of Glu^{126} (Fig. 1a). At these coordinates in the HCANNH spectrum we found the *intra*-residue Glu^{126} cross peak and its (^1H_N, ^{15}N) shifts (Fig. 1b). At the same time, we could cross check the (^1H_N, ^{15}N) shifts of Ser^{127}. Given the (^1H_N, ^{15}N) shifts of Glu^{126}, we could go back to the HCA(CO)NNH spectrum (Fig. 1c) and repeat the process until we reached either a glycine or proline residue (see below). In Fig. 2 the complementary assignment, working from the amino- to carboxy-terminus, is shown. In this case the assignment began at the ($^1H_\alpha$, $^{13}C_\alpha$) shifts of Thr^{124} in the HCA(CO)NNH spectrum. If we view the 2D (^1H_N, ^{15}N) plane, we obtain exclusively the *inter*-residue cross peak and the (^1H_N, ^{15}N) shifts of Val^{125} (Fig. 2d). At these coordinates in the HCANNH spectrum we found the *intra*-residue Val^{125} cross peak and its ($^1H_\alpha$, $^{13}C_\alpha$) shifts (Fig. 2c). Once again at the same time we could cross check the ($^1H_\alpha$, $^{13}C_\alpha$) shifts of Thr^{124}. Having found the ($^1H_\alpha$, $^{13}C_\alpha$) shifts of Val^{125}, we could go back to the HCA(CO)NNH spectrum and repeat the process (Fig. 2b). The information obtained from the assignment in opposite directions is clearly complementary, particularly for glycine and proline residues (see below) and serves as an internal cross check.

The assignment stops at glycine residues because the τ_1 delay (see Fig. 1 in Boucher et al., 1992b) was optimised for residues which have a single α-proton, i.e., those other than glycine. Assignments stop at proline residues because they have no amide protons. However, the assignment of the proline ($^1H_\alpha$, $^{13}C_\alpha$) shifts is possible when working in the carboxy- to amino-terminal direction and the assignment of glycine (^1H_N, ^{15}N) shifts is possible when working in the amino- to carboxy-terminal direction. To determine the ($^1H_\alpha$, $^{13}C_\alpha$) shifts of the glycine residues, we used a combination of 3D ^{15}N-edited TOCSY (Marion et al., 1989a) and 3D HCCH COSY spectra (Bax et al., 1990a) which we had previously acquired. An attractive alternative would be to record a 3D HCA(N)NH experiment with τ_1 optimised for glycine residues (1.7 ms). For larger proteins, it would be advantageous to record a 4D HNCAHA spectrum instead of a HCANNH spectrum. We have found that cross peaks for all residues, including the glycines (at low intensitiy), are present in the 4D HNCAHA spectrum, which minimises the number of discontinuities, but does not offer the possibility of spectral editing (Boucher et al., 1992b).

In principle, the complete assignment of backbone nuclei can be obtained using the strategy described here because it does not depend upon knowledge of side-chain type. In practice, due to overlap, intensity variations and breaks in the assignment at glycine and proline residues, it is very useful to be able to identify, at frequent intervals, the side-chain type for comparison with the primary sequence of the protein. Both 3D HCCH COSY or HCCH TOCSY spectra (Bax et al., 1990a,b) give ($^1H_\alpha$, $^{13}C_\alpha$) chemical shifts for unique spin systems (Gly, Ala, Val, Thr, Leu, Ile and Lys). The 3D HCA(N)NH spectrum could also be used to identify glycines (see above). Incorporation of ^{15}N labels into specific amino acids (Yamasaki et al., 1992) gives analogous unique (^1H_N,

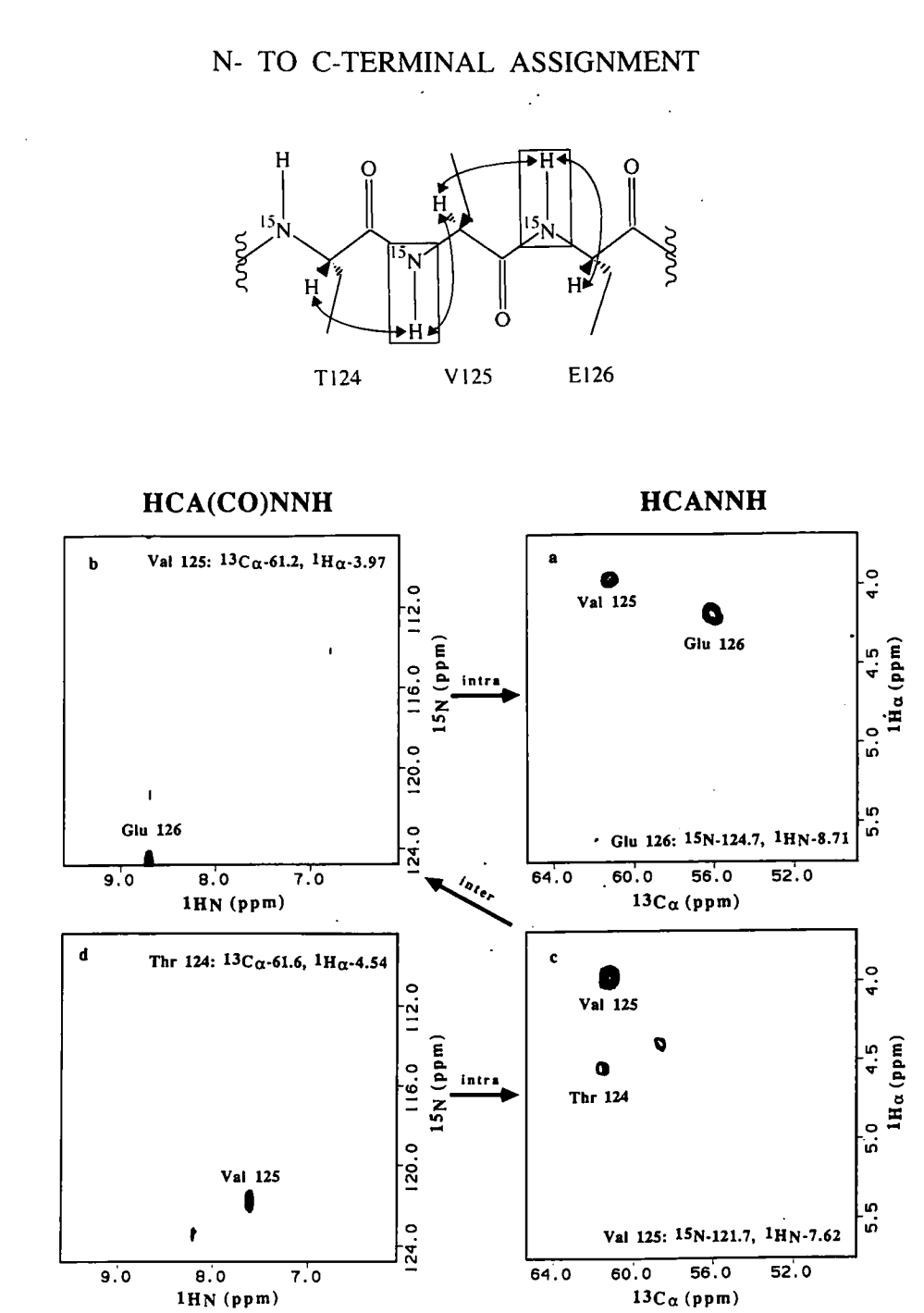


Fig. 2. The complementary amino- to carboxy-terminal assignment of the L8 loop of c-H-*ras* p21 (1–166).GDP; experimental details are as in Fig. 1. Panels (a) and (c) show specific sequential planes ($f_1 = {}^1H_\alpha$ and $f_2 = {}^{13}C_\alpha$) at particular ($f_3 = {}^{15}N$ and $f_4 = {}^1H_N$) chemical shifts from the HCANNH spectrum. Panels (b) and (d) show specific sequential planes ($f_3 = {}^{15}N$ and $f_4 = {}^1H_N$) at particular ($f_1 = {}^1H_{\alpha-1}$ and $f_2 = {}^{13}C_{\alpha-1}$ chemical shifts from the HCA(CO)NNH spectrum.

Fig. 3. A schematic representation of the sequential assignment obtained from the two 4D NMR experiments. Each X represents a pair of chemical shifts, either (^{1}H, ^{15}N) or ($^1H_\alpha$, $^{13}C_\alpha$), missing from both data sets. The only discontinuities, apart from the expected breaks which occur for glycine and proline, occurred at N86, T87, S127 and T158. The assignments contained in Table 1 (supplementary information) include the glycine ($^1H_\alpha$, $^{13}C_\alpha$) chemical shifts obtained from 3D experiments (see text).

^{15}N) chemical shifts. Although we relied mainly on the former approach, we found both to be useful and complementary.

The extent of the sequential assignments obtained from the 4D NMR data is summarised in Fig. 3 and the complete assignment including the glycine ($^1H_\alpha$, $^{13}C_\alpha$) chemical shifts is given in Table 1 (supplementary material). Discontinuities in the assignment occur primarily at glycine and proline residues, but occasionally also at some serine and threonine residues. Glycine, proline, serine and threonine spin systems can be easily identified in 3D HCCH COSY and TOCSY spectra, thereby providing cross checks. The extent to which the spin-system type was checked depended on a number of factors including the level of confidence in the connection, the ability to identify uniquely the amino-acid type using the 3D HCCH spectra as well as the correlation between the observed $^{13}C_\alpha$ chemical shift and the amino-acid type (Spera and Bax, 1991).

In conclusion, we have obtained a virtually complete sequential resonance assignment of c-H-*ras* p21 (1–166).GDP, thus providing the first step towards detailed studies of the structure, dynamics, mechanisms and interactions of *ras* proteins. We are currently completing the resonance assignments (^{1}H, ^{13}C) of the side chains as a prelude to structural studies of wild-type and oncogenic *ras* p21 complexed to GDP and GTP.

ACKNOWLEDGEMENTS

We thank Richard DeLoskey, James Krywko, Thomas Van Aken and Richard Yates for expert assistance. EDL thanks the Royal Society and St. John's College, Cambridge, for a travel grant. At Cambridge this work was supported by SERC and by Meiko Ltd.

Note added in proof

After this work was completed, Clubb et al. (1992) published their HNCAHA experiment. After submission, the HNCAHA experiment proposed by Wittekind et al. (1992) was also published by Kay et al. (1992).

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